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Phylogeny and distribution of protein kinase C variants in the zebrafish

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List of abbreviations

ALLG	anterior lateral line ganglion
CeP	cerebellar plate
CNS	central nervous system
CSG	cranial sensory ganglia
dn	dorsal neurons
DIL	diffuse nucleus of inferior lobe
DT	dorsal thalamus
EG	eminentia granularis
EmT	eminentia thalami
FG	facial ganglion
H	hypothalamus
Ha	habenula
INL	inner nuclear layer
LCa	lateral cerebellar area
MO	medulla oblongata
M1	migrated pretectal area
M2	migrated posterior tubercular area
M3	migrated area of EmT
OV	otic vesicle
P	pallium
pct	kidney proximal convoluted tubule
pf	pectoral fin
PLLG	posterior lateral line ganglion
Pr	pretectum
S	subpallium
T	tegmentum
TeO	optic tectum
TG	trigeminal ganglion
TLa	torus lateralis
TS	torus semicircularis
Va	valvula cerebelli
VG	vagal ganglion

VT ventral thalamus

1. Abstract

Conventional protein kinases – consisting of α, β, and γ family members – play key roles in numerous signal transduction events. Phylogenetic analysis demonstrated the existence of five *prkcs* (the genes representing PKCs) in zebrafish, two paralogous forms of *prkca* and -*b* and one *prkcg* variant. mRNA expression analysis showed distinct, mainly nervous system specific expression, for all five *prkc* genes. For *prkca* and *prkcb* paralogs prominent expression can be seen in the telencephalon, in diencephalic regions such as the habenula or the optic tectum, in hypothalamic areas and in distinct cerebellar structures.. Each transcript is additionally expressed in distinct areas: *prkcaa* is highly abundant in cranial sensory ganglia and in dorsal neurons of the hindbrain and the spinal cord, *prkcab* is strongly expressed in additional cerebellar regions, *prkcba* shows expression in the pectoral fin, the otic vesicle and in the proximal convoluted tubule of the kidney, and *prkcbb* shows prominent expression in different hypothalamic areas. Expression of *prkcg* is most striking in the cerebellum. As zebrafish PKCs are expressed in structures that are equivalent to mammals, the zebrafish model is well suited to study evolutionary conserved functions of PKCs in development and disease.

2. Introduction

The human genome comprises over 450 different protein kinases that regulate numerous cellular responses by phosphorylating protein substrates (Manning, Whyte, Martinez, Hunter & Sudarsanam, 2002). 10 of these protein kinases belong to the protein kinase C (PKC) family that evolved from the yeast PKC1 (Levin, Fields, Kunisawa, Bishop & Thomer, 1990; Watanabe, Chen & Levin, 1994). All PKCs share a highly conserved catalytic C-terminal domain which is linked to an N-terminal region containing regulatory modules (Newton, 2010). An intramolecular interaction of the regulatory and the catalytic region retains the enzyme in its inactive state which is terminated by binding of distinct second messengers to the regulatory domain (Newton, 2010). Depending on the second messengers that are capable of activating the enzyme and on structural similarities, PKCs are classified into three subfamilies: The classic or conventional PKCs α, β, and γ require diacylglycerol (DAG) along with phosphatidylserine (PS) and Ca^{2+} as cofactors for activation, whereas novel PKCs (δ, ε, θ and η) are activated by DAG and PS but independently of Ca^{2+}. Atypical PKCs (ζ and ι (human)/λ (murine)) are not activated by either DAG or Ca^{2+} but usually can be activated by PS or protein–protein interactions (Newton, 2010).

All PKCs, including conventional PKCs, belong to the family of serine-threonine kinases which primarily phosphorylate proteins involved in activation and inhibition of cellular processes. Thus, they play fundamental roles in numerous biological processes and are key therapeutic targets for many diseases, first and foremost for multiple forms of cancer (Kang, 2014) but also for neurological (Amadio, Battaini & Pascale, 2006; Zarate & Manji, 2009) or metabolic disorders (Clarke & Dodson, 2007; Das Evcimen & King, 2007), as well as diseases related to the immune (Lee, Duan & Tan, 2008) or the vascular system (Churchill, Budas, Vallentin, Koyanagi & Mochly-Rosen, 2008).

The zebrafish (*Danio rerio*) has emerged as a powerful model organism, even more so after the CRISPR/Cas9 system has revolutionized genome engineering (Li, Zhao, Page-McCaw & Chen, 2016). So far, the different PKC isoforms were localized in zebrafish by immunohistochemistry (Slatter, Kanji, Coutts & Ali, 2005; Patten, Sihra, Dhami, Coutts & Ali, 2007), however, few studies have investigated the role of conventional PKCs and their disease links in zebrafish (e.g. Williams, Feng, Martin & Poole, 2011). Moreover, all studies omit that the whole genome duplication event in the lineage of teleost fish could have led to more than one gene paralog for each mammalian ortholog (Amores et al., 1998; Vandepoele, Vos, Taylor, Meyer & Van de Peer, 2004; Glasauer & Neuhauss, 2014). So far, a proper

phylogenetic analysis to uncover gene paralogs in zebrafish and other vertebrates as well as the basic information of the distribution of specific zebrafish conventional PKCs are missing. Our phylogenetic analysis now revealed that all vertebrates possess α and β PKC orthologs but that the γ variant is absent from all birds we examined. In zebrafish, five *prkc* genes exist, of which two are gene paralogs resultant from the teleost specific whole genome duplication event. Moreover, we find distinct expression patterns for each *prkc,* mostly in homologous structures as their mammalian orthologs, suggesting evolutionary conserved functions that facilitate future comparative analysis across all vertebrate taxa.

3. Methods

Fish maintenance and breeding

Adult fish (RRID:ZIRC_ZL84) were maintained under standard conditions at 26 - 28°C in a 14-hour light/10-hour dark cycle. The wild-type strain WIK was used for all experiments described here. Embryos were raised at 28°C in E3 medium (5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, and 0.33mM $MgSO_4$). They were staged according to development in days post fertilization (dpf) and by morphological criteria (Kimmel, Ballard, Kimmel, Ullmann & Schilling, 1995). All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local authorities (Veterinäramt Zürich TV4206).

Annotation of *prkc* sequences

As gene predictions within GenBank are often produced by automated processes which have been shown to contain numerous errors, conventional *pkc* cDNA sequences used in this study were manually annotated. Sequences were identified and annotated using combined information from expressed sequence tags and genome databases (GeneBank, NCBI, http://www.ncbi.nlm.nih.gov, RRID: SCR_006472; Ensembl, http://www.ensembl.org/, RRID:SCR_002344). Human and mouse sequences were used as initial query (for more details on sequence annotation see Gesemann, Lesslauer, Maurer, Schönthaler, & Neuhauss, 2010). Using this procedure the coding sequences for the followings species were assembled and used for the phylogenetic analysis: Humans, *Homo sapiens,* hsa; mouse, *Mus musculus*, mmu; chicken, *Gallus gallus*, gga; green anole lizard, *Anolis carolinensis,* aca; chinese softshell turtle, *Pelodiscus sinensis;* western clawed frog, *Xenopus tropicalis*, xtr; coelacanth, *Latimeria chalumnae*, lch; zebrafish, *Danio rerio*, dre and the three spined stickleback, *Gasterosteus aculeatus,* gac. Ensemble gene numbers of the used sequences are listed in Table 1. Note that many of the above given genebank sequences are incomplete or contain multiple sequence errors. These sequences were manually corrected and completed. For each major clade, sequences from at least two additional species were analysed to confirm that the used species is indeed representative.

Phylogenetic tree analysis and sequence alignment

The phylogenetic analysis was performed on the Phylogeny.fr platform (http://www.phylogeny.fr, RRID:SCR_010266) (Dereeper et al., 2008) comprising the following steps: Sequences were aligned using MUSCLE (v3.8.31, https://www.ebi.ac.uk/Tools/msa/muscle/, RRID:SCR_011812) (Edgar, 2004) configured for highest accuracy (MUSCLE with default settings). Sequence length varied between 667 and 714 amino acids. After alignment, ambiguous regions (i.e. containing gaps and/or being poorly aligned) were removed using Gblocks (v0.91b) (Castresana, 2000) implementing the following parameters: The minimum length of a block after gap cleaning was set to 5; no gap positions were allowed in the final alignment; all segments with contiguous non-conserved positions larger than 8 were rejected; minimum number of sequences for a flank position was 55%. After curation 490 amino acids were chosen for further analysis. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1 aLRT, http://www.atgc-montpellier.fr/phyml/, RRID:SCR_014629) (Guindon & Gascuel, 2003). The default substitution model was selected assuming an estimated proportion of invariant sites (of 0.000) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 0.728). Reliability for internal branch was assessed using the aLRT test (Anisimova & Gascuel, 2006). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) and the svg file imported into CorelDRAW Graphics Suite (version x4; RRID:SCR_014235) for final editing.

Domain structures of the PKC proteins were determined using the embl SMART (Simple Modular Architecture Research Tool) program (http://smart.embl-heidelberg.de/). For alignment of whole proteins as well as to compare individual domains we used the blastp program. Alignments were verified by visual inspection and the alignment parameters were adapted (gap costs) to yield a minimum of sequence gaps.

Cloning of zebrafish *prkc* genes

Total mRNA was isolated from 5 day old WIK larvae using the QIAShredder and the RNeasy Mini kit (both Qiagen, Hombrechtikon, Switzerland) and further reverse transcribed to cDNA using the Superscript II Reverse Transcriptase kit (Invitrogen, Life Technologies, Zug, Switzerland) according to the manufacturer's instructions. To isolate the sequences of interest of each *prkc* gene, specific primers were designed (listed in Table 2) and used for polymerase chain reaction (PCR) with the Fast Cycling PCR kit (Qiagen). PCR products of appropriate size were purified with the Nucleo Spin Extract II kit (Macherey-Nagel,

Oensingen, Switzerland) and subcloned into the pCRII vector (TOPO TA-Cloning kit, Invitrogen). The resulting plasmids were transformed into TOP10 *Escherichia coli* cells and plasmid DNA from ON (overnight) grown cultures was isolated using the NucleoSpin Plasmid kit (Macherey-Nagel) or the Plasmid Mini kit (Qiagen). At least three independent clones were sequenced in house.

In situ hybridization (ISH)

The primers used for probe preparation are listed in Table 2. After cloning, plasmid purification and sequencing for confirmation (as described in Haug, Gesemann, Mueller & Neuhauss, 2013), plasmids were linearized with the appropriate restriction enzymes for T7 and Sp6, *in vitro* transcribed and the DNA was extracted with a standard phenol/chloroform protocol using pre-spinned RNase-free Phase-Lock tubes (5 Prime, Hamburg, Germany). The probes were DIG-labeled using a kit (DIG-RNA labeling kit; Roche, Rotkreuz, Switzerland) and applied on zebrafish larvae as previously described (Haug et al., 2013) at a concentration of approximately 2 ng/µl. Larvae were fixed at approximately 9 am. After successful staining, larvae were placed in 30% sucrose ON at 4°C, embedded in cryomatrix (Tissue Tek O.C.T., Sakura, Zoeterwonde, NL) and cut in 14 µm transverse sections using a microtome (HM 550, Microm). Sections were collected on glass slides and coverslipped with Kaiser's Glycerol Gelatine (Merck, Darmstadt, Germany). Images were taken with a light microscope (Olympus BX61). Adobe Photoshop (RRID:SCR_014199) and Illustrator CS5 were used to adjust brightness and contrast for proper arrangement.

4. Results

The zebrafish *prkc* gene family

In mammals, the family of conventional *prkcs* consists of the three members *a*, *b* and *g* (Newton, 2010). Based on sequence similarity, we identified and annotated cDNAs from major vertebrate clades, including mammals, birds, lizards, turtles, amphibians and fish. With the exception of avian species, which lack functional *prkcg* variants, all other analyzed classes still possess transcripts from *prkca*, *b* and *g* genes. However, due to the teleost specific genome duplication modern fish even harbor five different *prkc* genes, two paralogs for *prkca* and *b*, and one single *prkcg* gene (Fig. 1A). Overall sequence identity between zebrafish and human PKCα, β and δ proteins are in the range between 65 and 87%. However, sequence similarity of functional conserved amino acids is significantly higher ranging from 81 to 94% (Fig. 1B-F). Within the protein kinase domains (C1 and C2) and the catalytic domain homologies are even higher (Fig. 1B-F). We named the previously known zebrafish *prkca* as *prkcaa* and the newly annotated paralog *prkcab*. That these genes indeed originate from a whole genome duplication event, rather than from a local tandem duplication can be inferred from the different chromosomal location of the paralogous variants. While *prkcaa* is located on chromosome 6 *prkcab* can be found on chromosome 3. Different chromosomal locations can also be seen for the *prkcb* variants. *Prkcba* is located on chromosome 1 whereas *prkcbb* matches to chromosome 3, albeit to a completely different region than *prkcab*. Molecular cloning of the zebrafish coding sequences confirmed that all our annotated sequences are transcribed.

Expression pattern of *prkc* genes in larval zebrafish

To gain an overview of the abundance of zebrafish *prkc* transcripts we performed an expression analysis in 3- and 5 day old larvae by *in situ* RNA hybridization (Figs. 2-4). All family members show distinct expression patterns mostly in the central nervous system (CNS; for an overview see Table 3).

Both *prkca* paralogs are located in multiple regions of larval zebrafish CNS. Riboprobes constructed against *prkcaa* are highly abundant at both larval stages in the habenula (Ha), in cranial sensory ganglia (CSG: TG, ALLG, FG, PLLG, VG), in dorsal neurons (dn) of the hindbrain and the spinal cord, and in the retinal inner nuclear layer (INL; Fig. 2A-O,M1-6). Expression in CSG is best seen in the sections of 5 day old whole mount stained larvae (TG,

ALLG, FG, PLLG, VG; Fig. 2M4-6). While in both larval stages an additional weak expression of *prkcaa* was detected in the subpallium (S), expression in the pretectum (Pr) and in the cerebellum (Va, CeP; Fig. 2) was only found at 5dpf.

In contrast to a *prkcaa*, where the expression includes additional structures in 5 day old fish compared to 3 day old fish, the expression pattern of its paralog, *prkcab*, is alike at these two developmental stages. The transcripts of *prkcab* are located in the pallium (P), a variety of diencephalic (Ha, DT, M3, TLa, DIL) and mesencephalic structures (TeO, T), the cerebellum (Va, CeP, LCa, EG), the medulla oblongata (MO), and in the inner nuclear layer of the retina (INL; Fig. 2P-Z,Z1-5).

prkcba transcripts are broadly expressed throughout the CNS. Similar expression at both larval stages was found in the telencephalon (P, S), the habenula (Ha), the dorsal thalamus (DT), the eminentia thalami (EmT), in mesencephalic structures (TeO, T), , the cerebellar plate (CeP), and throughout the medulla oblongata (MO; Fig. 3A-H,H1-7). Expression in the valvula cerebelli (Va; Fig. 3H), the migrated part of the posterior

tubercular area (M2; Fig. 3H3), the pretectum (Pr; Fig. 3H3), and in the torus lateralis (TLa; Fig. 3H4-5) is only present in 5 day old larvae. Interestingly, we found *prkcba* expression outside the CNS in the pectoral fin at 3 dpf (pf; Fig. 3B,C) and very strongly in the otic vesicle (OV) and in the kidney proximal convoluted tubule (pct) in 5 day old larvae (Fig. 3F,G,H7).

Similar to *prkcba*, *prkcbb* transcripts are highly abundant in the pallium (P), in the eminentia thalami (EmT), and in parts of the cerebellum (Va, CeP, EG), and a lower expression is found in the midbrain (TeO, T; Fig. 3I-R,R1-7). While *prkcba* expression was restricted to the hypothalamic torus lateralis in 5 day old fish (TLa; Fig. 3H4-5), *prkcbb* is strongly expressed in a wider area of the hypothalamus (TLa, DIL; Fig. 3).

The last family member, *prkcg*, shows the most restricted expression pattern of all zebrafish *prkcs*. At both larval stages *prkcg* transcripts are highly expressed in the valvula cerebelli (Va) and the cerebellar plate (CeP; Fig. 4A-H,H4-5). In addition, a staining in the optic tectum (TeO) is also seen in 3- and 5 day old fish (Fig. 4A-H,H2-3).While at 3 dpf the expression is limited to the above mentioned structures, the 5 day old larvae show weak *prkcg* mRNA expression the pallium (P; Fig. 4H,H1), the migrated pretectal area (M1; Fig. 4G,H2), the hypothalamus (TLa, DIL; Fig. 4H3), and in an additional cerebellar structure, the eminentia granularis (EG; Fig. 4G,H5).

5. Discussion

In this study we analyzed the phylogeny of vertebrate conventional PKCs and determined the transcript distribution of zebrafish *prkcs* by RNA *in situ* hybridization. Zebrafish *prkcs* are preferentially expressed in the nervous system of larval zebrafish, mostly in homolog structures as their mammalian orthologs, suggesting evolutionary conserved functions. However, in zebrafish we annotated an additional member, *prkcab*, the paralog of *prkcaa*, and found two paralogs for *prkcb* and one *prkcg* gene.

The *prkc* gene family in zebrafish and other vertebrates

Conventional PCKs consist of the three different subtypes α, β and γ, that have a highly conserved sequence. While the α and β variants of mammals usually show amino acid identities in the range of 80% (similarities even go up to 90%), the γ variant has diverged somewhat more from the α and β paralogs showing identities of about 70%. Interestingly, we found *a* and *b prkcs* in all analyzed vertebrate species, however, the *g* paralog seems to be absent in birds. Nevertheless, we were able to identify remnants of former *prkcg* genes in several avian species; however, none of these seemed to be functional, probably only representing inactivated pseudogenes. Based on the occurrence of an additional round of whole genome duplication in teleost fish, we identified 5 *prkc* genes in modern fish. In the zebrafish, four of these genes have already been annotated, however, we found an additional zebrafish *prkc* gene, *prkcab*, the paralog of *prkca*. As the duplicated paralogs are located on different chromosomes within the genome, it seems likely that they indeed originate from the teleost-specific whole genome duplication (Amores et al., 1998; Vandepoele et al., 2004).

Diverse expression of *prkcs* in larval zebrafish

As we discovered a second paralog of *prkca* in zebrafish, and little knowledge about zebrafish *prkc* expression is available, we performed *in situ* hybridization for all *prkc* genes on whole mount larvae. All family members show distinct expression mostly in the nervous system at both analyzed larval stages (for an overview see Table 3). Overall, expression between 3 and 5 dpf changes only marginally but appears to be stronger in 5 day old larvae, suggesting that PKC function is more important at late developmental stages. The main function of conventional PKCs is the phosphorylation of proteins, thereby regulating a variety of signal transduction pathways (Newton, 2010). In 5 day old fish the initial steps of neuronal development are completed, as synapses are formed and signaling pathways are

established (Easter & Nicola, 1996; Schmitt & Dowling, 1999, Biehlmaier, Neuhauss & Kohler, 2003), Therefore a more prominent function at this later developmental stage is not surprising.as While the expression of paralogous genes is overlapping in some regions, other areas exclusively express the transcript of one paralog only. This is clearly seen for example for the two *prkcb* paralogs: While both genes are expressed in the pallium, the diencephalon/midbrain area, as well as in the cerebellum, *prkcba* shows a paralog-specific expression in the habenula, the medulla oblongata and the kidney proximal convoluted tubule, whereas *prkcbb* is expressed in additional hypothalamic and cerebellar areas. Such different but also overlapping expression for paralogous genes can be explained by various evolutionary events often occurring after a whole genome duplication (Force et al., 1999) such as the one observed at the base of the teleost lineage (Amores et al., 1998; Vandepoele et al., 2004). Accumulating mutations in regulatory regions of paralogous gene copies lead to sub- and/or neofunctionalization events, thereby placing selective pressure on both initially redundant paralogs (Force et al., 1999; Glasauer & Neuhauss, 2014).

prkc transcript expression in the zebrafish telencephalon

The mRNAs of *prkcab*, both *prkcb* paralogs and *prkcg* is located in the dorsal region of the pallium (P; Figs. 2,3,4), a region suggested to contain cell groups homologous to the amniote hippocampus (Rodríguez et al., 2002; Portavella, Vargas, Torres & Salas, 2002; Mueller, Dong, Berberoglu & Guo, 2011). Comparably, conventional PKCs are expressed in the mammalian hippocampus (Saito, Tsujino, Fukuda & Tanaka, 1994; Tejero-Díez, Rodríguez-Sánchez & Díez-Guerra, 1995) where they presumably are involved in long term spatial memory formation (Abeliovich et al., 1993; Paratcha et al., 2000; Huang, Huang, Wu & Boucheron, 2006; Bonini et al., 2007; Kim et al., 2012) and synaptic plasticity (Perez et al., 2001; Collingridge, Isaac & Wang, 2004). In zebrafish, learning and memory can be assessed by different experimental procedures (Roberts, Bill & Glanzman, 2013; Kalueff, Stewart & Gerlai, 2014). Up to date no link has been made between conventional PKC function and synaptic plasticity in zebrafish. However, as the evolutionary conserved expression of *prkcs* in zebrafish and mammals suggests an involvement in similar processes, zebrafish might be of great value for investigating PKC function in memory and learning.

Hypothalamic expression of *prkcs* in larval zebrafish

We find prominent expression of several *prkcs* in the larval zebrafish hypothalamic region. While the riboprobes of *prkcab* and *-bb* are highly expressed in both the torus lateralis (TLa)

and the diffuse nucleus of the inferior lobe (DIL), *prkcg* is expressed at a lower lever in both structures, and *prkcba* is only weakly expressed in the TLa but not in the DIL. Until now, PKC expression in the zebrafish hypothalamus has never been studied in detail. As growth hormone releasing hormone (GHRH) and GHRH like peptides (GHRH-LP), whose signaling pathway is known to involve conventional PKCs, have been detected in hypothalamic regions of teleosts (Pan, Lechan, Lin & Jackson, 1985; Xu, Roh, Loneragan, Pullar & Chen, 1999; Klausen, Severson, Chang & Habibi, 2005; Castro et al., 2009; Grey & Chang, 2011), expression of *prkcs* in this area are consistent with potential roles of PKCs in GHRH signaling. The hypothalamus is an evolutionary ancient and highly conserved key integrative center in the vertebrate brain which orchestrates multiple physiological functions. Compared to mammals, the zebrafish hypothalamus is structurally different from the mammalian one but its simple composition and the fact that it contains all the hypothalamic cell types makes it a suitable model for the study of hypothalamic function and signaling (Machluf, Gutnick & Levkowitz, 2011). Apart from the regulation of hormone release such as the above mentioned GH, the α form of the conventional PKCs is for example involved in the development of the hypothalamus (Choe, Lee & Kim, 2002) and in circadian rhythmicity (Nam et al., 2014), also a process that occurs in the hypothalamus. The model organism zebrafish could help understanding its role in the diverse neuronal circuits and facilitate the finding of new therapeutic targets.

prkc mRNA localization in the zebrafish cerebellum

Cerebellar Purkinje cells show a unique form of long term depression (LTD) that involves the metabotropic glutamate receptor 1 (mGluR1) as well as PKCα. PKCα has an important function in cerebellar LTD by regulating postsynaptic AMPA receptor internalization (Linden & Connor, 1991; Matsuda, Launey, Mikawa & Hirai,, 2000; Wang & Linden, 2000; Gundlfinger, Kapfhammer, Kruse, Leitges & Metzger, 2003; Leitges, Kovac, Plomann & Linden, 2004). Consistently, we find a very strong expression of both *prkca* paralogs in the larval zebrafish cerebellum (Fig. 2), indicating that both gene copies are still required within this structure. However, expression of *prkcaa* is weak and only seen at 5 dpf. Hence we expect *prkcab* to execute most of the function in the cerebellum. Additionally, we find a prominent expression of both *prkcb* paralogs in the cerebellar plate (CeP; *prkcba*), in the valvula cerebelli (Va) and the CeP (*prkcbb*; Fig. 3). Although the function of PKCβ in the cerebellum is not fully understood, its expression in this structure has been described in other vertebrates as well (Barmack, Qian & Yoshimura, 2000; Metzger & Kapfhammer, 2003).

It is not surprising that we also locate the zebrafish *prkcg* in the cerebellum (Fig. 4), as the process of AMPAR internalization seems to be influenced by PKCγ activity (Shuvaev et al., 2011) and PKCγ is known to have a crucial function in cerebellar synapse maturation (Kano et al., 1995). Although no double staining was performed, the expression strongly resembles immunolabeling with the Purkinje cell marker Parvalbumin 7 (Bae et al., 2009). Purkinje cells start developing from around 3 dpf on and their number increases drastically shortly thereafter (Hamling Tobias & Weissman, 2015), as was also observed for *prkcg* expression. Mutations in *prkcg* cause Purkinje cell degeneration in the neurodegenerative spinocerebellar ataxia type 14 disorder (Chen et al., 2003; Sakai, Saito & Seki, 2011). As PKCγ only exist as a single copy in zebrafish and the expression pattern strongly resembles the one in mammals (Saito & Shirai, 2002; Metzger & Kapfhammer, 2003), the zebrafish is perfectly suited for studying this disease and learn more about the function of PCKγ in Purkinje cell development.

Both *prkca* paralogs show prominent expression in the retinal INL

The signal transmitted from the eyes to the brain is already highly modulated within the retina. Bipolar cells provide the link between the light sensitive photoreceptors and the ganglion cells that connect the retina to the brain (Euler, Haverkamp, Schubert & Baden, 2014). PKCα is expressed in rod bipolar cells where it is involved in scotopic signal modulation at the first visual synapse (Kosaka, Suzuki, Morii & Nomura, 1998; Ruether et al., 2010; Rampino & Nawy, 2011; Xiong et al., 2015). Consistent with that we find a prominent expression of both *prkca* paralogs in the zebrafish INL where bipolar cells are located (Fig. 2M3,Z2). However, while *prkcaa* transcript expression is restricted to the medial INL, *prkcab* is weakly expressed all over the INL being expressed in a wider range of bipolar cells or even in more marginally located horizontal or amacrine cells. This points towards paralog specific functions, that have to be further elucidated. Additional expression and function for PKCs in other retinal cells have been described (e.g. Rodrigues & Dowling, 1990; Kolb & Zhang, 1997; Williams et al., 1997; Pinzon-Guzman, Zhang & Barnstable, 2011), however our analysis only revealed expression of *prkca* paralogs in the INL. Analysis of adult retinae using PKC antibodies revealed further results that are discussed in Haug et al. (submitted).

Additional expressions of zebrafish *prkc* transcripts

Previous studies described an expression of a commercially available PKCα antibody in the trigeminal ganglion of larval zebrafish as well as in Rohon-Beard (RB) cells (Slatter et al., 2005). We do not find any *prkc* transcript to be expressed in RB cells at 3 or 5 dpf, however *pkcaa* shows very prominent expression in cranial sensory ganglia at both larval stages (CSG; Fig. 2G,M). In addition, a paralog specific *prkcaa* expression in a subset of cells in the hindbrain and the rostral spinal cord (dn; Fig. 2E,H,I,O), which resemble the staining of noradrenergic sensory neurons as seen in Holzschuh et al., 2003, and Ackerman, Nakkula, Zirger, Beattie & Boyd, 2009. As described in Aplysia, PKCα is involved in regulating synaptic strength in sensory neurons (Wan et al., 2012), a function that may be preserved in zebrafish sensory neurons.

Interestingly, we find a very strong expression of *prkcba* in the larval zebrafish kidney proximal convoluted tubule (pct; Fig. 3F,G,H7), a structure homologous to the proximal tubules of the mammalian kidney (Wingert & Davidson, 2008). In diabetics, the activity of PKCs is upregulated in the vascular tissue including the renal glomeruli of the kidney. PKCβ has been shown to be a critical enzyme in diabetic kidney disease as hyperglycemia induced chronic activation of PKCβ is primarily responsible for abnormal changes in the kidney (Koya, 2014). Moreover, a PKCβ inhibitor has even been clinically tested as therapeutic target (Tuttle et al., 2005). The zebrafish is already a well established model in kidney research, as genetics of kidney development are shared between zebrafish and mammals (Drummond, 2003; McCampbell & Wingert, 2014). Our data support these findings and indicate a subfunctionalization event, implying that only *prkcba* adopted the function in the zebrafish kidney and *prkcbb* has lost it during evolution.

6. Conclusion

Phylogenetic analysis of zebrafish conventional *prkcs* identified two pairs of *prkca* and *prkcb* paralogs, and one single *prkcg* gene that lost its paralog during evolution. Detailed expression studies in larval fish demonstrated diverse expression of *prkc* members in the central nervous system, implicating their importance in diverse cellular processes. Overall, *prkc* transcripts were found in similar regions as in other vertebrates, arguing for evolutionary conserved functions amongst orthologous genes.

Competing interests

We have no competing interests.

Authors' contributions

MFH carried out the experiments, participated in the design of the study and drafted and edited the manuscript; MG conducted the phylogenetic analysis and edited the manuscript; MB helped with the *in situ* hybridization; SCFN participated in the design of the study, coordinated the study and edited the manuscript. All authors gave final approval for publication.

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Figure 1: Phylogenetic relationship and sequence comparison of conventional PKCs

Phylogenetic analysis included sequences from the two mammalian (*Homo sapiens* (hsa) and *Mus musculus* (mmu)) and two teleost species (*Danio rerio* (dre) and *Gasterosteus aculeatus* (gac)), as well as one representative of birds (*Gallus gallus* (gga)), lizzards (*Anolis carolinensis* (aac)), turtles (*Pelodiscus sinensis* (psi)), amphibians (*Xepous tropicalis* (xtr)), and coelacanths (*Latimeria chalumnae* (lch)). The different classes are indicated and color coded. Maximum likelihood analysis shows that zebrafish PKCs group with the corresponding vertebrate orthologs and that teleosts possess two paralogs of PKCα and PKCβ but only one PKCγ variant. Note that no functional PKCγ ortholog can be found in the genome of birds, but that rudimentary sequences matching PKCγ are still traceable in corresponding bird genomes (indicated by the asterisk). The human PKCδ was used for rooting the tree. The scale bar of 0.6 corresponds to 60% amino acid exchange.

Sequence comparison between zebrafish and human PKC proteins (B-F). The domain structure of the PKCs are indicated. C1 is the protein kinase C conserved region 1 (C1) domain (Cysteine-rich domain), C2 equals the protein kinase C conserved region 2 (CalB) and STK depicts the Serine/Threonine protein kinases, catalytic domain. Sequence identities and sequence conservation are shown. Highly conserved regions are given in red, medium conserved areas are depicted in violet/blue and sequence conservation below 50% is given in light blue. Sequence comparisons using the second zebrafish paralogs (PCKαb and PCKβb) are not included as the results are almost identical to the results seen for the first paralog. Note that conservation in the different spacer regions is quite low, whereas the conservation in the functional domains is high between all different PKCs.

Figure 2: Transcript expression of *prkca* paralogs in larval zebrafish

Expression of *prkcaa* in 3- (**A-I**) and 5 day old (**J-O**) whole mount zebrafish larvae and the respective cross sections as depicted in M (**M1-6**). Transcripts of *prkcaa* are found in the telencephalon (S, Ha), in cranial sensory ganglia (CSG:TG,ALLG,FG,VG, PLLG) as well as in the inner nuclear layer of the retina (INL) and in dorsal neurons in the hindbrain and the spinal cord (dn) at both larval stages. In addition, an expression in the pretectum (Pr) and in the cerebellum (Va, CeP) is found at 5 dpf (L-N,M3,M5).

Expression of *prkcab* in 3- (**P-T**) and 5 day old (**U-Z**) whole mount zebrafish larvae and the corresponding cross sections as depicted in Z (**Z1-5**). We find *prkcab* mRNA to be located in a variety of regions throughout the telencephalon (P), diencephalon (Ha, M3, DT, TLa, DIL), midbrain (TeO), and hindbrain (cerebellar regions (Va, CeP, LCa, EG) and medulla oblongata (MO)), as well as in one retinal layer (INL). At both larval stages strongest expression is seen in the pallium (P), the optic tectum (TeO), in the hypothalamus (TLa, DIL) and the cerebellum (Va, CeP, LCa, EG). Expression between 3- and 5 day old fish does not change but appears stronger and more specific in most regions. For abbreviations, see list. All scale bars = 50 µm. Scale bar in H applies to A-D and F-H; I applies to E and I; O applies to J-O; T applies to P-T; Z applies to U-Z; Z5 applies to M1-6 and Z1-5.

Figure 3: Transcript expression of *prkcb* paralogs in larval zebrafish

Expression of *prkcba* in 3- (**A-D**) and 5 day old (**E-H**) whole mount zebrafish larvae and the corresponding cross sections as depicted in H (**H1-7**). Most prominent labeling of *prkcba* mRNA at both larval stages is found in the pallium (P), in diencephalic/mesencephalic regions (DT, TeO), the otic vesicle (OV), the cerebellar plate (CeP) and in the medulla oblongata (MO). Additionally, at 3 dpf transcripts are abundant in the pectoral fin (pf), and at 5 dpf in the hypothalamic torus lateralis (TLa), in the proximal convoluted tubule of the kidney (pct), and very weakly in the habenula (Ha) and in the valvula cerebelli (Va). Expression of *prkcbb* in 3- (**I-M**) and 5 day old (**N-R**) whole mount zebrafish larvae and the presumptive cross sections as depicted in R (**R1-7**). Both larval stages show a strikingly similar expression pattern of *prkcbb* transcripts in the pallium (P), the eminentia thalami (EmT), the optic tectum (TeO), the hypothalamus (TLa, DIL), and the cerebellum (Va, CeP). Additional lower expression is found in the torus semicircularis (TS) of the midbrain and in the cerebellar eminentia granularis (EG). For abbreviations, see list. All scale bars = 50 µm. Scale bar in M applies to A-D and I-M; R applies to E-H and N-R; R7 applies to H1-7 and R1-7.

Figure 4: Transcript expression of *prkcg* in larval zebrafish

Expression of *prkcg* in 3- (**A-D**) and 5 day old (**E-H**) whole mount zebrafish larvae and the respective cross sections as depicted in H (**H1-5**). At 3- and 5 dpf, *prkcg* transcripts are found in only few brain areas: in the optic tectum (TeO) and in the cerebellum (Va, CeP, EG). In 5 day old fish, *prkcg* transcripts are additionally weakly expressed in the pallium (P), in the diencephalic area of M1 and in hypothalamic regions (TLa, DIL). For abbreviations, see list. All scale bars = 50 µm. Scale bar in H applies to A-H; H5 applies to H1-5.

Table 1: Ensemble gene numbers used for phylogenetic analyis.

Table 1: Ensemble gene numbers used for phylogenetic analyis.

Species	Gene	Ensembl Nr.
Humans	*prkca*	ENSG00000154229
Homo sapiens (hsa)	*prkcb*	ENSG00000166501
	prkcg	ENSG00000126583
	prkcd	ENSG00000163932
Mouse	*prkca*	ENSMUSG00000050965
Mus musculus (mms)	*prkcb*	ENSMUSG00000052889
	prkcg	ENSMUSG00000078816
Chicken	*prkca*	ENSGALG00000025950
Gallus gallus (gga)	*prkcb*	ENSGALG00000037943
Lizard	*prkca*	ENSACAG00000000629
Anolis carolinensis (aca)	*prkcb*	ENSACAG00000002203
	prkcg	ENSACAG00000016368
Turtle	*prkca*	ENSPSIG00000016217
Pelodiscus sinensis (psi)	*prkcb*	ENSPSIG00000004330
	prkcg	no link
Frog	*prkca*	ENSXETG00000017489
Xenopus tropicalis (xtr)	*prkcb*	ENSXETG00000017294
	prkcg	ENSXETG00000010034
Coelacanth	*prkca*	ENSLACG00000004445
Latimeria chalumnae (lch)	*prkcb*	ENSLACG00000002827
	prkcg	ENSLACG00000007559
Zebrafish	*prkcaa*	ENSDARG00000039241
Danio rerio (dre)	*prkcab*	ENSDARG00000099841
	prkcba	ENSDARG00000008723
	prkcbb	ENSDARG00000022254
	prkcg	ENSDARG00000004561
Stickleback	*prkcaa*	ENSGACG00000018519
Gasterosteus aculeatus (gac)	*prkcab*	ENSGACG00000011652
	prkcba	ENSGACG00000016775
	prkcbb	ENSGACG00000014351
	prkcg	ENSGACG00000010646

Table 2: Primer pairs used for full length cloning and riboprobe preparation.

Table 2: Primer sites used for full-lenght cloning and riboprobe preparation.				
Full length cloning		**RNA probes**		
Gene	**Sequence 5'-3'**	**Gene**	**Sequence 5'-3'**	**Clone length**
prkcaa_dr_0005s	CTGATACACAAAGCAACGAG	prkcaa_fwd	GGACTCATACACCAAGGAATG	602 bp
prkcaa_dr_1995as	GGAGGGATGGACAAATTG	prkcaa_rev	GACCCACTGTCATCCAAAG	
prkcab_dr_0000s	ATGGCTGATCATCTGATACAG	prkcab_fwd	CTGCGACAGAAGAACGTG	986 bp
prkcab_dr_1979as	TCGGGGTTTATGAAGGAG	prkcab_rev	CATCACCTTCCCAAAACTC	
prkcba_dr_0013s	TCGGATTCAGATGGCAAG	prkcba_fwd	TCGGATTCAGATGGCAAG	1017 bp
prkcba_dr_1962as	GCCTCGAAATTCCTCTTG	prkcba_rev	GCACCATGATGAAGTTGAAG	
prkcbb_dr_0018s	CAGCGATGGAGAGGAGAG	prkcbb_fwd	TGACCACTGCATGATGAAC	683 bp
prkcbb_dr_1992as	AGGGAATTCAGGGTTTATG	prkcbb_rev	TTCCTCTCAGCCAACATTAC	
prkcg_dr_start_s	GTGAAGGACCACCAGTTTAC	prkcg_fwd	GTGAAGGACCACCAGTTTAC	944 bp
prkcg_dr_end_as	ATTCCTCTTGATTTATTGCTG	prkcg_rev	ATGAGGAAGTTGAAATCGTG	

Table 3: Overview of *prkc* mRNA expression in zebrafish

Region / Gene		Telence-phalon		Diencephalon/Midbrain							Hindbrain						other
		P	S	Ha	Thalamus		TeO	T	Hypo-thalamus		other regions	Cerebellum				MO	other regions	
					DT	VT			TLa	DIL		Va	CeP	LCa	EG			
prkcaa	3d	–	+	++	–	–	–	–	–	–	–	–	–	–	–	–	++ CSG + dn	++ retinal INL
	5d	–	+	++	–	–	–	–	–	–	± Pr	+	+	–	–	–	++ CSG + dn	++ retinal INL
prkcab	3d	+	–	+	+	–	+	±	+	+	+ M3	+	+	+	+	±	–	± retinal INL
	5d	+	–	+	+	–	+	±	+	++	+ M3	++	++	++	+	±	–	+ retinal INL
prkcba	3d	++	±	±	+	–	+	+	–	–	± EmT	–	++	–	–	++	–	+ OV + pf
	5d	++	±	+	+	–	+	+	+	–	± EmT, ± M2 + Pr	±	++	–	–	++	–	+ OV ++ pct
prkcbb	3d	++	–	–	–	–	+	+	+	+	++ EmT + TS	++	++	–	±	–	–	–
	5d	++	–	–	–	–	+	+	++	++	++ EmT + TS	++	++	–	±	–	–	–
prkcg	3d	–	–	–	–	–	±	–	–	–	–	++	+	–	–	–	–	–
	5d	+	–	–	–	–	+	–	+	+	+ M1	++	++	–	+	–	–	–

– no expression; ± weak expression; + medium expression; ++ strong expression

Expression data is shown for 3- and 5 day old fish. See list for abbreviations.

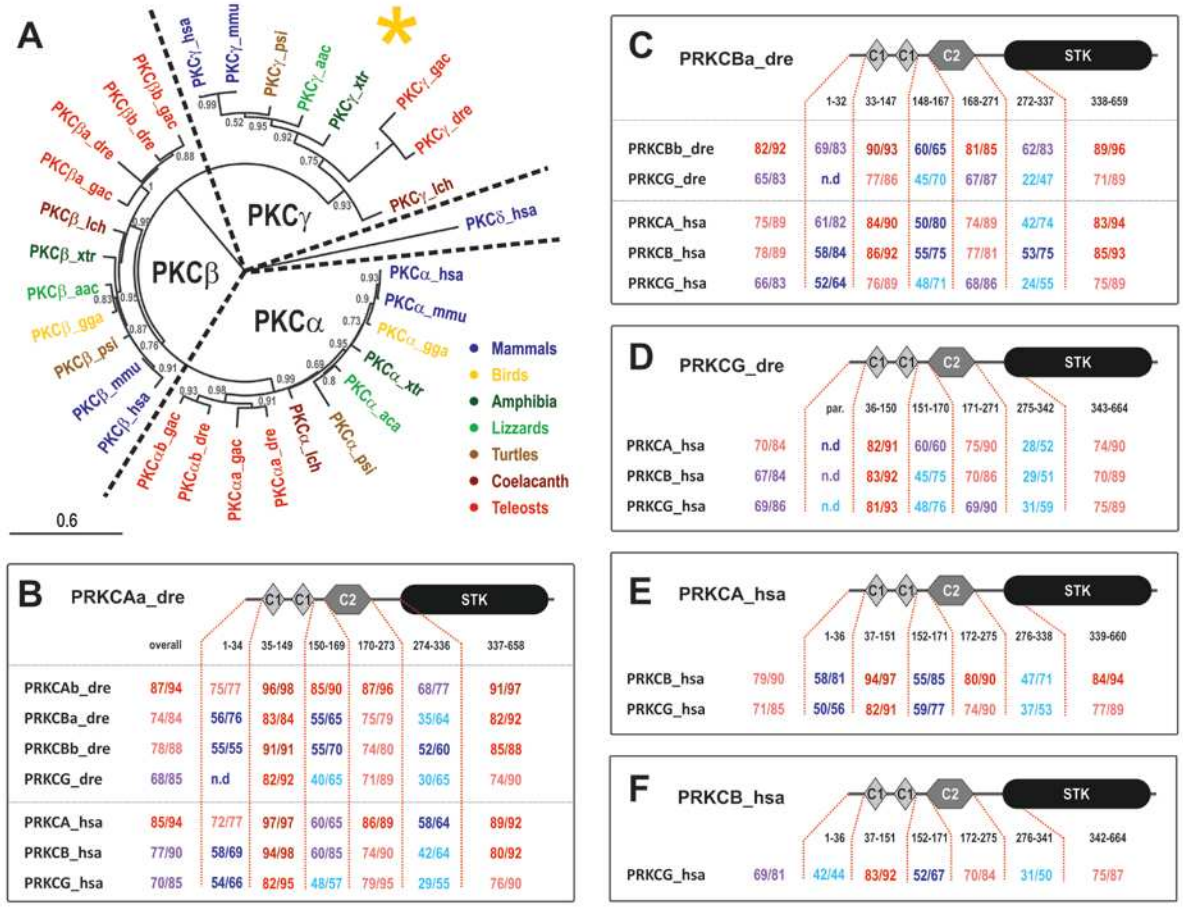

Figure 1

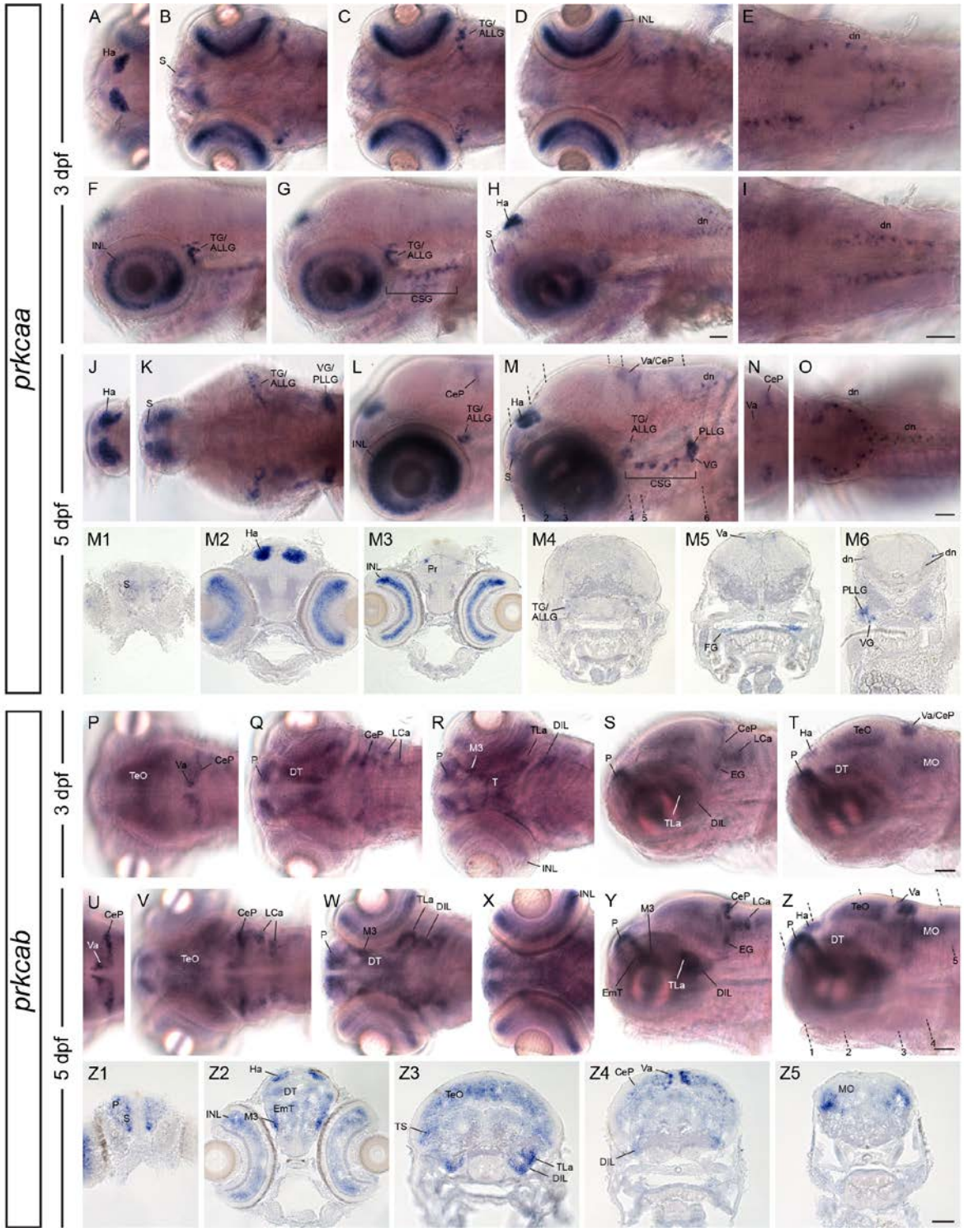
prkcaa
3 dpf
5 dpf
A
Ha
B
S
C
TG/ ALLG
D
INL
E
dn
F
INL
TG/ ALLG
G
TG/ ALLG
CSG
H
Ha
S
dn
I
dn
J
Ha
K
S
TG/ ALLG
VG/ PLLG
L
CeP
TG/ ALLG
INL
M
Va/CeP
dn
Ha
TG/ ALLG
PLLG
VG
S
CSG
1 2 3 4 5 6
N
CeP
Va
O
dn
cn
M1
S
M2
Ha
M3
INL
Pr
M4
TG/ ALLG
M5
Va
FG
M6
dn
dn
PLLG
VG
prkcab
3 dpf
5 dpf
P
TeO
Va
CeP
Q
P
DT
CeP
LCa
R
P
M3
T
TLa
DiL
INL
S
P
CeP
LCa
EG
TLa
DiL
T
Ha
P
TeC
Va/CeP
DT
MO
U
CeP
Va
V
TeO
CeP
LCa
W
P
M3
DT
TLa
DiL
X
INL
Y
P
M3
CeP
LCa
EG
EmT
TLa
DiL
Z
Ha
P
TeO
Va
DT
MO
5
1 2 3 4
Z1
P
S
Z2
Ha
DT
EmT
INL
M3
Z3
TeO
TS
TLa
DiL
Z4
CeP
Va
DiL
Z5
MO

Figure 2

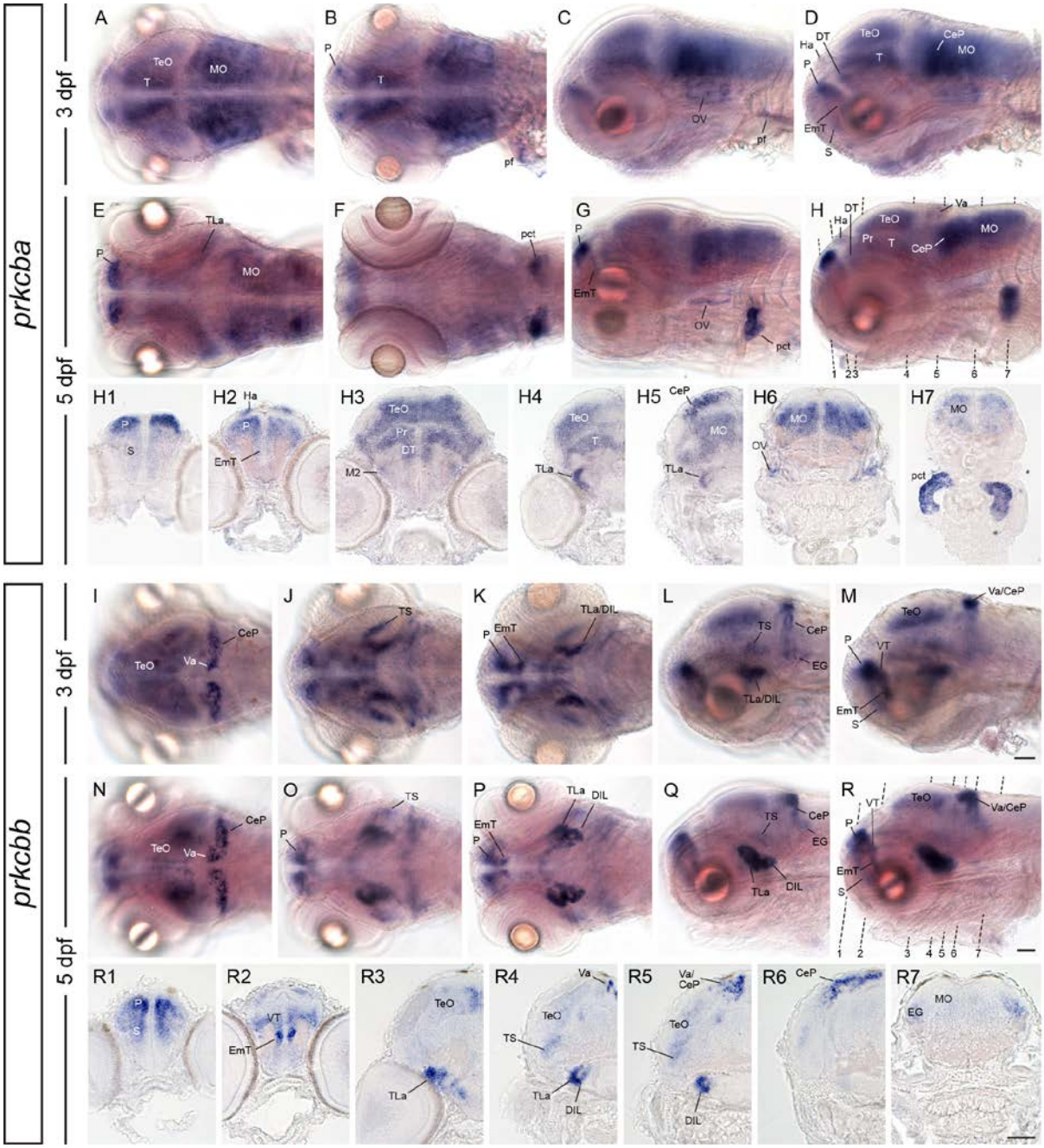
prkcba
prkcbb
3 dpf
5 dpf
A
B
C
D
E
F
G
H
H1
H2
H3
H4
H5
H6
H7
I
J
K
L
M
N
O
P
Q
R
R1
R2
R3
R4
R5
R6
R7
TeO
MO
T
P
pf
OV
DT
Ha
CeP
Va
EmT
S
TLa
pct
Pr
M2
TS
TLa/DIL
EG
VT
DIL
Va/CeP

Figure 3

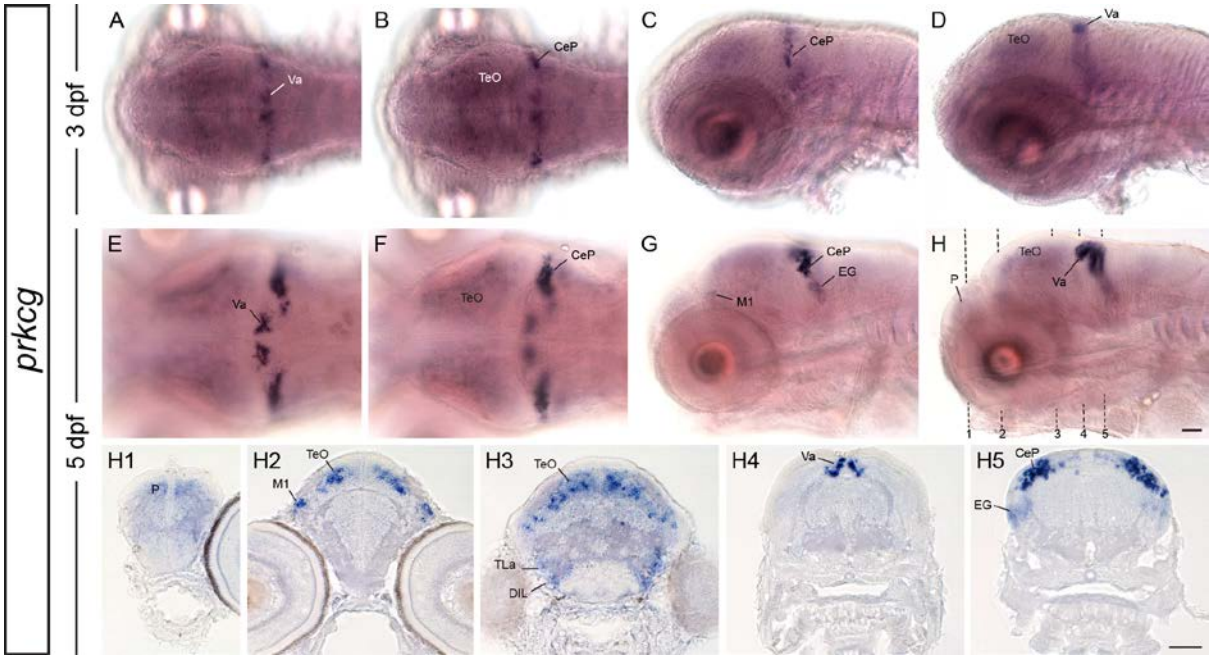
prkcg
3 dpf
5 dpf
A
Va
B
TeO
CeP
C
CeP
D
Va
TeO
E
Va
F
TeO
CeP
G
CeP
EG
M1
H
TeO
Va
P
1
2
3
4
5
H1
P
H2
TeO
M1
H3
TeO
TLa
DIL
H4
Va
H5
CeP
EG

Figure 4